EFFECT OF VARIOUS CARBON AND NITROGEN SOURCES ON THE GROWTH AND SPORULATION OF *CLAVICEPS MICROCEPHALA*

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Abstract

The carbon and nitrogen nutrition of *Claviceps microcephala*, isolated from the ear heads of *Pennisetum typhoides*, was studied at 24°C for 20 days at pH 6·0. Out of forty-one carbon compounds tested, the pathogen showed excellent growth on dextrose, sucrose, pectin and methyl alcohol; good on mannose, fructose and maltose; fair on galactose, coconut oil and isopropyl alcohol; and poor or no growth on rest of the carbon compounds. Out of thirty-three nitrogenous compounds tested, the pathogen showed excellent growth on casein hydrolysate, yeast extract, asparagine, peptone, proline, glutamic acid and aspartic acid; good on ammonium nitrate and ammonium oxalate; fair on ammonium chloride, ammonium sulphate, arginine mono HCl and ammonium phosphate; and poor or no growth on rest of the nitrogen compounds. In general, compounds which supported best mycelial growth of *C. microcephala* yielded its excellent sporulation and *vice versa*.

Introduction

Claviceps microcephala (Wallr.) Tul. causes a serious disease of hybrid crops of *Pennisetum typhoides* (Burm.) Stapf. & Hubbard. The diseased material was collected from the various fields of Punjab and Haryana states during the months of October and November, 1967. Several monosporic isolates of *C. microcephala* were made separately on potato dextrose agar slants (peeled and sliced potatoes 200 g, dextrose 20 g, agar 20 g, and distilled water 1,000 ml). No marked morphological variation among its various isolates was observed. The present paper deals with the influence of different carbon and nitrogen compounds on the growth and sporulation of a representative isolate of *C. microcephala*. Such studies have not been carried out previously on this organism.

MATERIAL AND METHODS

Nutritional studies were carried out with a basal medium containing dextrose 20 g, asparagine 3·740 g, KH_2PO_4 10 g, $MgSO_4$ $7H_2O$ 0·250 g, $Fe_2(SO_4)_3$ $6H_2O$ 0·005 g, and distilled water 1,000 ml. This basal medium was found to give excellent growth of *C. microcephala* in the preliminary studies. Dextrose and asparagine of the basal medium were replaced by various carbon* and nitrogen** sources to yield 8 g carbon and 693 mg nitrogen per litre. Twenty-five millilitres of medium were poured in 100 ml Pyrex glass Erlenmeyer flasks and three replicates were taken in each case. Initial pH of the various media was adjusted after sterilization to pH 6·0. However, the initial pH of the solution of separate carbon compounds was adjusted to 7·0 (neutral) to minimize any breakdown of these during autoclaving while the combined solution of remaining ingredients of basal medium was sterilized in autoclave without adjusting to the optimum pH. A carbon solution and remaining ingredients of basal medium after autoclaving were mixed together proportionally to get the normal solution of the basal medium and pH was adjusted to 6·0. The spore suspension was prepared in sterilized distilled water from 6 days old culture. The medium in each flask was inoculated by 1 ml standardised spore suspension (8–16 spores per low power field of the compound microscope). It may be mentioned here that pH 6·0, temperature 24° C and incubation period of 20 days were found to be optimum conditions for the growth of *C. microcephala* by preliminary experiments. The cultures were filtered and dried at 60°C to a constant weight in hot air oven for 24 hours. The data were recorded in terms of final pH, sporulation and dry weight of the mycelium.

For the estimation of spore concentration in different experiments, each culture flask was shaken and the fungal growth was mixed thoroughly by means of glass rod in order to get a homogenous mixture. After shaking thoroughly, drops were taken from this homogenate and the spore count was estimated under low power of a compound microscope. The degree of sporulation was measured on the basis of average number of spores present per low power field of the compound microscope and recorded in the following grades :

* Soluble starch, pectin and inulin were added at the rate of 20 g each, while oils were added at the rate of 20 ml each per litre of the basal medium.

** Peptone, yeast extract and casein hydrolysate were added at the rate of 5g each per litre of the basal medium.

Number of spores per low power field of the microscope	Degree of sporulation	Symbol used
No spore	Nil	..
1–10	Poor	+
10–20	Fair	++
20–30	Good	+++
Above 30	Excellent	++++

The growth of the fungus on different carbon and nitrogen sources has been termed excellent, good, fair and poor on the basis of following dry mycelial weights :

Category	Dry weight, mg
Excellent	Above 150
Good	100–150
Fair	50–100
Poor	Below 50

Experimental Work

Carbon nutrition.—A total of sixteen carbohydrates comprising of three pentoses, five hexoses, four disaccharides, one trisaccharide and three polysaccharides were used as the sole source of carbon on the growth and sporulation of *C. microcephala.* The data on dry weight, sporulation and final pH are summarised in Table I. *C. microcephala* showed excellent growth on dextrose, sucrose and pectin; good on mannose, fructose and maltose; fair on galactose; poor on lactose, starch, raffinose, melibiose, arabinose, inulin, xylose and ribose; and no growth on sorbose. Sporulation was excellent on dextrose, fructose, sucrose, maltose and pectin; good on lactose; and poor or no sporulation on rest of the carbohydrates.

TABLE I

Effect of different carbon compounds used singly as the sole source of carbon on the growth and sporulation of C. microcephala *after* 20 *days of incubation at* 24°C *Initial pH adjusted to* 6·0.

Carbon source	Dry wt in mg	Sporulation	Final pH
Control (without carbon)	0	..	6·0
Carbohydrates			
D (+) Xylose	15	..	5·8
L (+) Arabinose	20	+	6·3
D (−) -Ribose	15	..	5·5
Dextrose	300	++++	6·4
D (−) -Fructose	136	++++	6·3
D (−) Mannose	141	..	5·9
D (+)-Galactose	75	+	6·2
L-Sorbose	0	..	6·0
Sucrose	278	++++	6·0
Lactose	40	+++	7·0
D-Maltose	102	++++	6·9
D (+) Melibiose	22	..	6·6
Raffinose	30	..	6·1
Inulin	20	..	6·7
Starch	31	..	6·9
Pectin	210	++++	6·6

Sorbose is utilised poorly or not at all by the fungi, in general. It has been reported not only unutilisable for many fungi but also toxic for several others. The toxicity is evidenced by the death of hyphal tips followed by meagre branching of the mycelium below the killed portion (Lilly and Barnett,

1953). It has been suggested that probably this sugar interferes with the respiratory pathway of the microorganisms. In order to find out whether the absence of growth of *C. microcephala* on sorbose is due to its inhibitory effect or nonutilisation, an experiment was set up as follows:

Equivalent amount of a carbon source so as to furnish 8 g/l of carbon, which is present in 20 g/l of dextrose, was added to basal medium containing sorbose. The mixture of two carbohydrates, therefore, furnished twice the amount, *i.e.*, equivalent to 40 g dextrose per litre. A control with 40 g dextrose was set up to study if there was any adverse effect on the growth of *C. microcephala* due to increased amount of total sugars. For the sake of comparison, the pathogen was also grown on individual sugars. Twenty-five millilitres of medium were poured in each flask and three replicates were taken in each case. Rest of the procedure was as usual. The data on dry weight, sporulation and final pH are summarised in Table II. It is apparent from the

TABLE II

Effect of sorbose used singly and in combination with other carbon sources on the growth and sporulation of C. microcephala *after* 20 *days of incubation at* 24° *C. Initial pH adjusted to* 6·0.

Carbon source	Dry wt in mg	Sporulation	Final pH
Dextrose 40 g/l	320	++++	6·4
Sorbose	0	..	6·0
Dextrose	306	++++	6·4
Dextrose + Sorbose	26	..	6·0
Fructose	136	++++	6·3
Fructose + Sorbose	16	..	6·0
Mannose	144	..	5·9
Mannose + Sorbose	15	..	6·2
Sucrose	278	++++	6·0
Sucrose + Sobrose	23	..	6·0
Maltose	102	++++	6·9
Maltose + Sorbose	12	..	6·2
Pectin	206	++++	6·6
Pectin + Sorbose	146	..	6·2

TABLE III

Effect of different alcohols, oils and organic acids used singly as the sole source of carbon on the growth and sporulation of C. microcephala *after* 20 *days of incubation at* 24°C. *Initial pH adjusted to* 6·0.

Carbon source	Dry wt in mg	Sporulation	Final pH
Control (without carbon)	0	—	6·0
Alcohols			
Mannitol	45	—	7·3
Dulcitol	25	—	6·9
Methyl alcohol	166	—	6·8
Ethyl alcohol	37	—	7·2
Isopropyl alcohol	50	—	6·6
n-Butyl alcohol	11	—	6·0
Oils			
Castor oil	0	—	6·0
Olive oil	0	—	6·0
Cotton seed oil	0	—	6·0
Almond oil	0	—	6·0
Coconut oil	64	++++	6·7
Organic acids			
Formic acid	0	..	6·0
Acetic acid	0	..	6·0
Propionic acid	0	..	6·0
Butyric acid	0	..	6·0
n-Velaric acid	0	..	6·0
Stearic acid	0	..	6·0
Lactic acid	0	..	6·0
Oxalic acid	0	..	6·0
Maleic acid	0	..	6·0
Succinic acid	0	..	6·0
Sebacic acid	0	..	6·0
Malic acid	0	—	6·0
Tartaric acid	20	—	6·0
Citric acid	24	—	6·0

data that the growth of *C. microcephala* was markedly inhibited by sorbose even in the presence of dextrose, fructose, mannose, sucrose and maltose and to some extent only in the presence of pectin. Thus, for this fungus sorbose is definitely an inhibitory carbon agent and did not allow the utilisation of these sugars which wer , otherwise, good carbon sources for its growth.

Six alcohols, five oils and fourteen organic acids were used as the sole source of carbon for the growth and sporulation of *C. microcephala.* The data on dry weight, sporulation and final pH summarised in Table III showed excellent growth on methyl alcohol; fair on coconut oil and isopropyl alcohol; poor on mannitol, ethyl alcohol, dulcitol, citric acid, tartaric acid and *n*-butyl alcohol; and no growth on rest of the carbon sources. The pathogen showed excellent sporulation on coconut oil and no sporulation on rest of the carbon sources.

Nitrogen nutrition.—Eight inorganic and five organic nitrogenous compounds were used as the sole source of nitrogen on the growth and sporulation of *C. microcephala.* The data on dry weight, sporulation and final pH summarised in Table IV showed excellent growth on casein hydrolysate, yeast extract,

TABLE IV

Effect of different inorganic and organic nitrogenous compounds used singly as the sole source of nitrogen on the growth and sporulation of C. microcephala *after* 20 *days of incubation at* 24°C. *Initial pH adjusted to* 6·0.

Nitrogen source	Dry wt in mg	Sporulation	Final pH
Control (without nitrogen)	0	—	6·0
Potassium nitrate	40	—	6·0
Potassium nitrite	0	—	6·0
Sodium nitrate	40	—	6·0
Ammonium oxalate	105	+	5·0
Ammonium sulphate	90	+++	6·0
Ammonium nitrate	112	+++	4·8
Ammonium chloride	99	+++	4·8
Ammonium phosphate	86	++	5·6
Urea	30	—	6·3
Asparagine	305	++++	6·3
Peptone	305	++++	5·8
Yeast extract	332	++++	6·0
Casein hydrolysate	335	+	5·8

asparagine and peptone; good on ammonium nitrate and ammonium oxalate; fair on ammonium chloride, ammonium sulphate and ammonium phosphate; and poor growth on potassium nitrate, sodium nitrate and urea. The pathogen showed excellent sporulation on asparagine, peptone and yeast extract; good on ammonium sulphate, ammonium nitrate and ammonium chloride; fair on ammonium phosphate; poor on ammonium oxalate and casein hydrolysate; and no sporulation on potassium nitrate, sodium nitrate and urea. However, there was neither any growth nor any sporulation on nitrite of potassium.

H-ion concentration is known to influence markedly the utilisation of KNO_2 by different fungi. Therefore, an experiment was set up to find out the effect of whole range of pH on the growth and sporulation of *C. microcephala* on KNO_2. It is clear from Table V that this pathogen showed neither growth on acidic range nor on alkaline range.

TABLE V

Effect of different hydrogen-ion concentration on the utilization of potassium nitrite for the growth and sporulation of C. microcephala *after* 20 *days of incubation at* 24°C.

Initial pH	Dry wt in mg	Sporulation	Final pH
2·0	0	—	2·0
3·0	0	—	3·0
4·0	0	—	4·0
5·0	0	—	5·0
6·0	0	—	6·0
7·0	0	—	7·0
8·0	0	—	8·0
9·0	0	—	8·2
10·0	0	—	8·8
11·0	0	—	8·9

Twenty amino acids were tested as the sole source of nitrogen on the growth and sporulation of *C. microcephala.* The data on dry weight, sporulation and final pH are summarised in Table VI. *C. microcephala* showed excellent growth on proline, glutamic acid and aspartic acid; fair on arginine

TABLE VI

Effect of different amino acids used singly as the sole source of nitrogen on the growth and sporulation of C. microcephala *after* 20 *days of incubation at* 24°*C. Initial pH adjusted to* 6·0.

Nitrogen source	Dry wt in mg	Sporulation	Final pH
Control (without nitrogen)	0	..	6·0
Glycine	46	+++	6·0
DL-iso-Leucine	0	..	6·0
L-Leucine	0	..	6·0
B-alanine	0	..	6·0
DL-Valine	0	..	6·0
DL-nor-Valine	0	..	6·0
DL-Threonine	16	..	6·1
DL-Serine	13	..	5·9
L-Proline	223	..	5·7
L-Cystine	0	..	6·0
DL-Methionine	0	..	6·0
L-Arginine mono HCl	88	..	5·7
L-Lycine mono HCl	10	..	5·9
DL-Aspartic acid	202	+++	6·7
L-Glutamic acid	224	+++	7·0
DL-Phenylalanine	0	..	6·0
L-Tyrosine	0	..	6·0
DL-Tryptophane	0	..	6·0
L-Histidine monohydrochloride	12	..	5·8
DL-Histidine dihydrochloride	14	..	5·8

mono HCl; poor on glycine, threonine, histidine dihydrochloride, serine, histidine mono HCl and lysine mono HCl; and no growth on rest of the amino acids. The pathogen showed good sporulation on glycine, aspartic acid and glutamic acid and no sporulation on rest of the amino acids.

Cystine did not support any growth of *C. microcephala* and, therefore, it was thought desirable to see whether it is a poor source of nitrogen or it is inhibitory to the growth and sporulation of this fungus. For this the following experiment was set up :

Equivalent amount of an amino acid so as to furnish 693 mg/l of nitrogen, which is present in 3·740 g/l of asparagine, was added to the basal medium containing cystine. The mixture of two amino acids, therefore, furnished twice the amount of nitrogen, *i.e.*, equivalent to 7·480 g/l of asparagine. Twenty-five millilitres of the basal medium were poured in each flask and three replicates were taken in each case. Rest of the procedure was as usual. The data on dry weight, sporulation and final pH are summarised in Table VII, which showed that cystine completely inhibited the growth of *C. microcephala* when used in combination with good nitrogen sources, *i.e.*, proline, aspartic acid and glutamic acid.

TABLE VII

Effect of cystine used singly and in combination with other amino acids on the growth and sporulation of C. microcephala *after* 20 *days of incubation at* 24°*C. Initial pH adjusted to* 6·0.

Nitrogen source	Dry wt in mg	Sporulation	Final pH
Cystine	0	..	6·0
Proline	225	..	5·7
Proline + cystine	0	..	6·0
Aspartic acid	206	+++	6·7
Aspartic acid + cystine	0	..	6·0
Glutamic acid	226	+++	7·0
Glutamic acid + cystine	0	..	6·0

Discussion

Pentoses usually do not support good growth of fungi. Similarly, xylose, arabinose and ribose supported poor growth of *C. microcephala* studied here. Poor growth on pentoses may be due to their inability to enter the phosphogluconate oxidation pathway as pentose phosphate.

Dextrose, sucrose, mannose, fructose and maltose supported excellent or good growth of *C. microcephala*. Similarly, a very large number of fungi have been reported to make excellent or good growth on these carbon sources. On the other hand, it showed fair growth on galactose and poor on lactose, raffinose and melibiose. Lilly and Barnett (1951), after studying 57 fungi, characterised lactose as a poor carbon source for their growth. Many other workers have also reported it to be a poor source of carbon for the growth of fungi. Galactose, raffinose and melibiose are well utilised by some fungi but poorly by others.

Sorbose, a ketohexose, did not support any growth of *C. microcephala*. It has been reported not only unutilisable for many fungi but toxic for others. Lilly and Barnett (1953) mentioned that sorbose may be stimulatory in the presence of glucose and inhibitory in the presence of sucrose and maltose. They attributed it to the size and complexity of sugar molecules. In the present investigations, however, the growth of *C. microcephala* was markedly inhibited by sorbose in the presence of dextrose, fructose, mannose, sucrose and maltose and to some extent in the presence of pectin. Similarly, Matsushima and Klug (1958) found that the growth of *Ustilago maydis* line 10 A^4 was completely inhibited by sorbose in the presence of maltose, arabinose, galactose and raffinose. Murray and Andrian (1960) also found that sorbose severely inhibited the growth of *Neurospora crassa* (wild type) in the presence of maltose, arabinose, galactose and fructose.

Utilisation of higher complex carbohydrates by fungi may largely depend upon their synthesis of suitable hydrolytic enzyme. According to Lilly and Barnett (1951), only those fungi which produce amylase are able to utilise starch; this ability is common but not universal among fungi. Starch has been found to be an excellent source of carbon for a large number of fungi studied so far by various workers. However, it supported poor growth of *C. microcephala* studied here. Some other fungi have been reported to utilize poorly or not at all this polysaccharide, such as *Penicillium digitatum* (Fergus, 1952), *Cercospora hibiscina* and *C. withaniae* (Thind and Mandahar, 1964),

Cercospora arachidicola (Landers, 1964) and *Alternaria tenuis* (Singh and Khanna, 1966). Inulin supported poor growth while pectin supported excellent growth of *C. microcephala*. Inulin, generally, is a poor source of carbon for the growth of many fungi. On the other hand, pectin generally serves as an excellent source of carbon for the growth of many fungi, such as *Cephalothecium roseum* (Thind and Madan, 1967), *Ustilago nuda tritici* (Sen and Munjal, 1968) and *Linderina pennispora* and *L. macrospora* (Chan and Stephen, 1968).

Alcohols, oils and organic acids in general are reported to be poor sources of carbon for the growth of fungi. This pathogen showed fair growth on coconut oil and isopropyl alcohol; poor on mannitol, ethyl alcohol, dulcitol, and *n*-butyl alcohol; and no growth on rest of the carbon sources. However, methyl alcohol supported excellent growth of *C. microcephala* studied here. This result is quite interesting because this alcohol is reported to be a poor source of carbon for the growth of all other fungi studied by various workers. To our knowledge there is no report of any fungus showing good or excellent growth on methyl alcohol.

C. microcephala showed poor sporulation with arabinose and no sporulation with xylose and ribose. Similarly, pentoses, generally are known to be poor sources of carbon for the sporulation of fungi. However, excellent or good sporulation on xylose and arabinose has been reported for *Colletotrichum gloeosporioides* (Tandon and Chandra, 1962) and *Fusarium solani* and *Botryodiplodia ananassae* (Bhargava, 1971).

Dextrose, fructose, sucrose, maltose and pectin supported excellent sporulation of *C. microcephala*. All these carbohydrates have been reported to show excellent or good sporulation of fungi, in general. Lactose, generally, is a poor source of carbon for the sporulation of fungi. However, it showed good sporulation of this pathogen. Same is true of *Curvularia penniseti* (Agarwal, 1958), *Alternaria citri* (Hasija, 1970) and *Curvularia pallescens* (Bais *et. al.*, 1970). *C. microcephala* supported poor sporulation on galactose and no sporulation on mannose, sorbose, melibiose, raffinose, inulin and starch. Out of these galactose, mannose, melibiose, raffinose and starch have generally been reported to support excellent or good sporulation of many fungi.

C. microcephala yielded excellent sporulation on coconut oil and no sporulation on all other alcohols, oils and organic acids. In general, all these

carbon compounds are poor sources for the growth as well as for the sporulation of fungi.

Nitrate-nitrogen has been reported to be well utilised by a large majority of fungi. However, *C. microcephala* made poor growth on potassium and sodium nitrates. There are some fungi which are either unable to utilize nitrates or utilize them extremely poorly, such as *P. digitatum* (Fergus, 1952), *Schizophyllum commune* (Swack and Miles, 1960), *Thraustochytrium* spp. (Goldstein, 1963), *Phallus ravenelii* and *Crucibulum levis* (Howard and Howard, 1969) and *Althornia crouchii* and *Ostracoblabe implexa* (Alderman and Jones, 1971).

Nitrites are generally toxic on the acidic pH range (Cochrane, 1950 and 1958; Cochrane and Conn, 1950; Lilly and Barnett, 1951) while some fungi show growth on it in the alkaline medium. However, potassium nitrite was found to inhibit the growth of *C. microcephala* on the acidic as well as on alkaline range.

C. microcephala made fair to good growth on various ammonium salts used. Many of the fungi are reported to use ammonium nitrogen well.

Urea supported poor growth of *C. microcephala* as is true of *Pleospora indica* (Mandahar, 1965), *Colletotrichum coccodes* (Kurtz and Fergus, 1964) and *F. solani* and *B. ananassae* (Bhargava, 1970). However, Agarwal (1958) and Thind and Madan (1969) have observed good growth of *C. penniseti* and *C. roseum*, respectively, on urea. Asparagine supported excellent growth of the present fungus and similarly, a very large number of fungi have been reported by various workers to make excellent or good growth on it. However, there are some fungi which grow poorly on it, such as *Leptomitus lacteus* (Schade, 1940), some Hymenomycetes (Yusef, 1953) and *Zygorhynchus* spp. (Sarbhoy, 1965). Casein hydrolysate, yeast extract and peptone all supported excellent growth of the present fungus. These are reported to be excellent or good sources of nitrogen for the growth of fungi, in general.

The amino acid requirement for the growth of *C. microcephala* appears to be very specific since the organism made excellent growth only on proline, aspartic acid and glutamic acid. Arginine mono HCl supported fair growth of the organism while poor or no growth was observed on rest of the amino acids tested here. Cystine completely inhibited the growth of *C. microcephala* when used in combination with good nitrogen sources, *i.e.*, proline, aspartic

acid and glutamic acid. Similarly, Lewis (1957) has reported cystine to be a constant inhibitor for the growth of *Alternaria solani*.

Potassium nitrate and sodium nitrate, which are poor sources of nitrogen for the growth of *C. microcephala* did not yield any sporulation. Conversely, ammonium nitrate, a good supporter of growth, yielded good sporulation of this fungus. However, ammonium oxalate, a good supporter of growth, induced poor sporulation while ammonium sulphate and ammonium chloride, which are fair sources of nitrogen for the growth of this pathogen, showed good sporulation. Ammonium phosphate, a fair supporter of growth, also yielded fair sporulation.

Asparagine, peptone and yeast extract, which are excellent sources of nitrogen for the growth of *C. microcephala*, also yielded excellent sporulation. However, casein hydrolysate, which is a good supporter of growth, induced poor sporulation. On the other hand, urea which is a poor source of nitrogen for the growth of this pathogen, showed no sporulation.

Aspartic and glutamic acids both are excellent sources of nitrogen for the growth of *C. microcephala* and they proved to be good for its sporulation as well. However, glycine, which is a poor supporter of growth, yielded good sporulation.

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